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Low dose irinotecan improves advanced lupus nephritis in mice potentially by changing DNA relaxation and anti-dsDNA binding

Running title: Low dose irinotecan as a new therapeutic option for SLE

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Competing interests

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Abstract

Objective Albeit clear advances in the treatment of SLE, many patients still present with refractory lupus nephritis requiring new treatment strategies for this disease. Here we determined whether reduced doses of the topoisomerase I inhibitor irinotecan, which is known as chemotherapeutic agent, were able to suppress SLE in NZB/W F1 mice. We further evaluated the potential mechanism how irinotecan influenced the course of SLE.

Methods NZB/W F1 mice were treated with low dose irinotecan either from week 24 of age or from established glomerulonephritis defined by a proteinuria \geq grade 3+. Binding of anti-dsDNA antibodies was measured by ELISA; and DNA relaxation was visualized by gel electrophoresis.

Results Significantly reduced irinotecan dosages improved lupus nephritis and prolonged survival in NZB/W F1 mice. The lowest dose successfully used for the treatment of established murine lupus nephritis was more than 50 times lower than the dose usually applied for chemotherapy in humans. As a mechanism, low dose irinotecan reduced B cell activity; however, the levels of B cell activity in irinotecan-treated mice were similar to those in Balb/c mice of the same age suggesting that irinotecan did not induce a clear immunosuppression. In addition, incubation of double-stranded (ds) DNA with topoisomerase I increased binding of murine and human anti-dsDNA antibodies showing for the first time that relaxed DNA is more susceptible to anti-dsDNA antibody binding. This effect was reversed by addition of the topoisomerase I inhibitor camptothecin.

Conclusion Our results propose topoisomerase I inhibitors as a novel and targeted therapy for SLE.

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease mainly affecting women of childbearing age. It is estimated that in the United States up to 275,000 adult women suffer from SLE (1). The disease involves different organs but immune complex glomerulonephritis is a major cause of morbidity and mortality (2). Due to the application of immunosuppressive drugs, the survival of patients with lupus-associated glomerulonephritis increased from a 5-year survival of 44% in the 1950s to a 10-year survival of 88% recently (3). Despite these advances in the treatment of SLE, the life expectancy of patients with lupus and renal damage was recently demonstrated to be 23.7 years shorter compared to the general population (4).

Unselective immunosuppressive drugs remain the central strategy to control lupus nephritis. Medication consists of an induction therapy with cyclophosphamide and prednisolone or mycophenolate mofetil followed by a maintenance therapy with azathioprine or mycophenolate mofetil (5, 6). Major side effects of this medication are infections and they bear the risk of malignancies whereupon cyclophosphamide also causes amenorrhea (7, 8). Interestingly, despite clear beneficial effects in human disease, in mice none of these agents alone or in combination, were shown to reverse established proteinuria (9).

New hope for the treatment of lupus patients came up with the development of so called biologicals. Recently, belimumab, an antibody directed against BAFF, a B cell survival factor, was approved for the treatment of human SLE. While a phase III trial reached statistical significance in lupus patients without active glomerulonephritis, belimumab showed a “number needed to treat” of nearly 11 (10). Moreover, a second phase III trial demonstrated moderate clinical effects in patients with SLE only when using belimumab

with higher doses and only at week 52 while there was no statistical significance at week 76 despite continuous treatment (11). Other biologicals, such as rituximab or ocrelizumab which are antibodies designed to deplete B cells, either failed to show an improvement of renal function in a recent phase III trial treating patients with proliferating lupus nephritis (12) or were stopped due to the rate of infections (13). Collectively, despite progress in research, the treatment of active lupus nephritis remains an unsolved problem requiring new therapeutic approaches.

Our group previously reported that the topoisomerase I inhibitor irinotecan suppressed lupus nephritis and significantly prolonged survival in NZB/W F1 mice (14), a model of spontaneous SLE (15, 16). This finding was made by serendipity and was entirely new since enzymatic topoisomerase I activity had been not implicated in the pathogenesis or treatment of SLE thus far. The function of this ubiquitously expressed protein is the reduction of torsional stress that develops during replication of DNA. To realize this, topoisomerase I binds to supercoiled genomic DNA and induces transient single-stranded (ss) DNA breaks followed by stress-relieving rotation of the nicked DNA strand (DNA relaxation). Then, ssDNA breaks are re-ligated by topoisomerase I to reconstitute intact double-stranded (ds) DNA (17). Inhibitors of topoisomerase I bind to the topoisomerase I-DNA complex (and not to DNA or to topoisomerase I alone) which is also named the cleavable complex, thereby preventing the re-ligation of DNA (18). As a consequence, at least two different scenarios are known for cells treated with an inhibitor of topoisomerase I. In dividing cells with enhanced DNA replication the complex of DNA, topoisomerase I and its inhibitor can collide with DNA replication forks existing only during S-phase. Collision of the cleavable complex with DNA replication forks results in the generation of irreversible dsDNA breaks followed by the induction of cell death (19, 20). This is how inhibitors of topoisomerase I induce cell death (apoptosis) in proliferating cancer

tissue and explains why the topoisomerase I inhibitor irinotecan is approved for the treatment of colorectal malignancies (21). Contrary, in non-dividing cells treatment with topoisomerase I inhibitors results in the production of ssDNA, which is believed to reduce cell's replication capacity but which is not lethal (22). Using the topoisomerase I inhibitor irinotecan for the treatment of murine lupus nephritis, we proposed the second scenario since our previous data clearly demonstrated an inhibition of apoptosis instead of apoptosis induction in the kidneys of irinotecan-treated lupus-prone mice (14). However, our suggestion to have found a potential new treatment option for SLE was not acknowledged mainly due the topoisomerase I inhibitor's problems as a chemotherapeutic agent (2, 23). In the present study we intended to investigate whether reduced dosages of irinotecan are still able to suppress lupus nephritis. We further determined whether immunosuppression, changed DNA sensing processes or an altered binding of anti-dsDNA antibodies is the leading mechanism in irinotecan-mediated suppression of SLE.

Material and Methods

Patient sera

Blood sera which are positive for anti-dsDNA antibodies were obtained from lupus patients at Hannover Medical School (Hannover, Germany) and at Medizinische Klinik und Poliklinik IV, University of Munich (Munich, Germany). Sera collection was approved by the institutional review boards and written informed consent was obtained from all patients.

Animals

Six weeks old female NZB/W F1 mice were obtained from Jackson Laboratories, Balb/c mice used as control were supplied by Charles River. Animals were kept in isolated ventilated cages. All experiments were performed with the agreement of the Kantonale Tierversuchskommission (Bern, Switzerland). Directly after arrival mice were randomized to the respective groups with 5 animals per cage.

Animal study I: prevention of SLE using low dose irinotecan

From week 19 of age all mice were monitored for proteinuria and body weight once a week. n=10 per group. Treatment with irinotecan (Campto®, Pfizer) started at week 24 of age and was conducted *i.p.* thrice a week with doses of 3.125, 6.25, 12.5 or 25 mg/kg (i.e. 9.4, 18.8, 37.5, or 75 mg/kg per week, respectively). The control group received saline with 10 ml/kg body weight. Treatment was repeated every fourth week. The beginning of the disease was defined as two instances of proteinuria grade 4+ one week apart. Criteria for sacrifice due to the severity of the disease were proteinuria grade 4+ (≥ 2000 mg/dl) and a weight loss of >25% calculated from the beginning of the disease. Proteinuria was measured with Albustix® (Siemens

Healthcare Diagnostics) and quantified as grade 0 (negative), grade 1+ (≥ 30 mg/dL), grade 2+ (≥ 100 mg/dL), grade 3+ (≥ 300 mg/dL) and grade 4+ (≥ 2000 mg/dL) according to manufacturer's recommendations. The experiment was terminated at week 65. Separate groups of mice ($n=14$) were sacrificed after one and three weeks after completion of the second treatment cycle (at week 29 and week 31 respectively). As control groups NZB/W F1 mice from week 8 of age ($n=20$) and from week 23 of age ($n=14$) as well as Balb/c from week 30 of age ($n=10$) which had received no treatment were used.

Animal study II – treatment of established lupus nephritis with low dose irinotecan

Treatment of established lupus nephritis was performed as described before (14) with slight modifications. NZB/W F1 mice ($n=10$ per group) were measured for proteinuria and body weight once a week starting at week 16 of age. The beginning of the disease was defined as two instances proteinuria \geq grade 3+, one week apart. Treatment with irinotecan was started at the onset of the disease and performed with 1 mg/kg i.p. thrice a week for one week (3 mg/kg per week). The control group received saline with 10 ml/kg. After one treatment cycle no further treatment was applied for at least three weeks. Relapsing disease was defined as two instances of proteinuria \geq grade 3+, one week apart. The treatment of relapsing disease was performed as the initial treatment. Criteria for sacrifice were the same as described above.

Isolation of splenocytes

Spleen was isolated, transferred into ice-cold 2% FCS/PBS and smashed on a sterile grid with a pestle. Cells were re-suspended and debris was allowed to settle out by

centrifugation for 2 min at 65 x g, 4°C. Subsequently, splenocytes were isolated by centrifugation at 290 x g and 4°C for 5 min. Cells were resuspended in RPMI-1640, supplemented with 5% FCS and adjusted to 10^7 cells/ml after checking for viability by trypan blue exclusion.

Flow cytometry

Splenocytes (10^6 per sample) were incubated with Fc receptor-blocking monoclonal antibody (clone 2.4G2, BD Biosciences) for 30 min, followed by incubation with fluorochrome-labelled monoclonal antibodies for 30 min on ice. Cells were measured on a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo™ software (Tree Star). Anti-CD4 (clone RM4-5), anti-CD8a (clone 5H10), anti-Ly6C/G (clone RB6-8C5), anti-CD69 (clone H1.2F3), and anti-CD45R/B220 (clone RA36B2) were obtained from Caltag Laboratories.

ELISpot assay

Serial dilutions of splenocytes (2×10^5 – 1.25×10^4 /well) in RPMI-1640 medium, supplemented with 5% FCS were added to 96-well MultiscreenHTS Immobilon-P-bottomed plates (Millipore), coated with goat anti-mouse IgG (Fc-specific, Sigma) antibody. After 4 h at 37°C plates were washed and incubated for 1 h with alkaline phosphatase-conjugated anti-mouse IgG (H+L chain specific, Southern Biotech). Spots were developed with BCIP/NBT plus substrate (Sigma) and counted using an ELISpot reader (Autoimmun Diagnostika).

Antibody ELISAs

Total IgGs and anti-dsDNA antibodies were determined as described before (14).

Quantitative PCR

Total RNA was extracted from splenocytes using the GenElute Mammalian Total RNA Kit (Sigma). Quantitative PCR was performed as described before (24) using the TaqMan™ Assay-on-Demand™ assay kit Mm00439546_s1 (Applied Biosystems) for murine IFN- β .

Preparation of nucleosomal DNA

Nucleosomal DNA was isolated using the EZ Nucleosomal DNA Prep Kit (Zymo Research). Briefly, nuclei from mouse splenocytes were isolated and digested with Atlantis dsDNase. After spin-column purification the quality of the nucleosomal preparation was checked by resolving the DNA on a 2% agarose gel.

Effect of topoisomerase I and camptothecin on binding of anti-dsDNA antibodies

Modification of DNA using topoisomerase I alone or in combination with the topoisomerase I inhibitor camptothecin was performed essentially as described before (22). Fifty μ g of filtered calf thymus (ct) dsDNA, nucleosomal DNA or AT-rich dsDNA Poly(dA:dT) (Sigma) were incubated with different concentrations of recombinant human topoisomerase I (Creative Biomart) using 0.9, 2.8, 8.3, 25, 75 or 225 ng per μ g DNA in a 1 ml reaction containing 40 mM Tris, pH 7.5, 100 mM KCl, 10 mM MgCl₂, 0.5 mM DTT, 0.5 mM EDTA and 30 μ g/ml BSA (Sigma) for 30 min at 37°C. Then 384-well Nunc 'maxisorp' plates were coated with 30 μ l per well over night at 4°C. Plates were blocked with PBS containing 1% Casein (Pierce) for 1 h. Plasma samples from NZB/W F1 mice (n=10) or human serum samples (n=14) were diluted 1: 500 in PBS and incubated for 1 h. In some experiments the monoclonal

anti-ds DNA antibody HYB331 (Abcam) was used at a concentration of 1 $\mu\text{g/ml}$. Bound antibodies were detected as described before for anti-dsDNA ELISA.

To evaluate the effect of the topoisomerase I inhibitor camptothecin, 50 $\mu\text{g/ml}$ of calf thymus dsDNA were incubated with 75 ng recombinant topoisomerase I per μg DNA and the indicated amounts of camptothecin (Sigma) directly on 384-well Nunc 'maxisorp' plates for 1 h at 37°C. 10% DMSO was used as control. Determination of anti-dsDNA binding was assessed as described above.

DNA relaxation assay

Fifty $\mu\text{g/ml}$ of DNA supercoiled pBR322 plasmid DNA (Inspiralis) was incubated with the indicated concentrations of topoisomerase I for 1h at 37°C. DNA relaxation was visualized by electrophoresis using a 1% agarose gel.

Statistical analysis

Data are expressed as the mean \pm SEM and analyzed using either one-way or two-way analysis of variance followed by Bonferroni's *post hoc* test. Some data were assessed with Kruskal-Wallis test. Survival data were analyzed using the Mantel-Cox log-rank test. For all tests the software GraphPadPrism version 6.0 was used. Differences were considered statistically significant at *P* values <0.05.

Results

Reduced dosages of irinotecan suppress lupus nephritis in NZB/W F1 mice

The original experimental plan for reduced dosages of irinotecan in lupus-prone mice was designed to end up with a dose which is no more clinically active. This approach would have enabled us to correlate functional effects such as a potential irinotecan-induced immunosuppression with clinical data in order to find out the mechanism of irinotecan-mediated suppression of murine SLE. Treatment of NZB/W F1 mice with irinotecan was started at 24 weeks of age. At this time point, mice already possess signs of lupus such as elevated anti-dsDNA antibody titer (25). The highest dose of irinotecan was 25 mg/kg per injection which was given thrice a week (e.g. 75 mg/kg per week). This amount comprised the lowest irinotecan dose successfully used in previous experiments (14). Moreover, this dose corresponds to half of the dose which is used for chemotherapy in humans (for conversion of dosages from mice to human see below). Doses for other groups were divided in half. According to this schedule the lowest irinotecan dose used for these experiments was 3.125 mg/kg per injection or 9.376 mg/kg per week corresponding to a dose which is more than 15 times lower than the dose used for chemotherapy of metastatic cancer. Surprisingly, even the lowest irinotecan dose demonstrated a suppression of lupus nephritis as shown by a significant reduction in proteinuria for all irinotecan-treated groups from week 40 until week 52 of age. After week 53 of age only dosages of ≥ 6.25 mg/kg irinotecan significantly diminished the grade of proteinuria (**Fig. 1A**). The body weight of saline-treated control mice started to decline after week 35 of age what was attributed to severely diseased animals. In contrast, the body weight of all irinotecan-treated groups remained stable until week 58 of age and showed a significant difference to saline-treated mice from week 42 until week 64 of age (**Fig. 1B**). Mice were sacrificed

due to the severity of the disease which was defined as proteinuria grade 4+ (≥ 2000 mg/dL) and a weight loss of $>25\%$ calculated from the beginning of the disease. Subsequent analysis of survival data revealed a significantly prolonged survival for all irinotecan-treated groups compared to saline-treated control mice (**Fig. 1C**).

Low dose irinotecan does not induce immunosuppression

Beside the clinical effects of low dose irinotecan on lupus nephritis in NZB/W F1 mice we were interested whether these dosages still mediate immunosuppression. Previous experiments of our group had demonstrated that although irinotecan at higher dosages caused a transient down-regulation of B cell activity, the levels of anti-dsDNA antibodies were not decreased (14). We sacrificed lupus-prone mice one and three weeks after the second cycle of treatment with irinotecan (e.g. at 29 and 31 weeks of age). Untreated NZB/W F1 mice 8 and 23 weeks of age and Balb/c mice 30 weeks of age were used as controls. Determination of spleen weight revealed no significant differences between saline- and irinotecan-treated mice at 29 weeks of age. Furthermore, only NZB/W F1 mice which received 12.5 and 25 mg/kg irinotecan exhibited a lower spleen weight compared to age-matched Balb/c mice (**Fig. 2A**). At 31 weeks of age there was a significant reduction of spleen weight in mice treated with 6.25 and 25 mg/kg irinotecan compared to the saline-treated group, but no difference between saline-treated mice and the other irinotecan-treated groups (**Fig. 2A**). Moreover, no differences were seen in the number of B cells splenocyte population both in the groups sacrificed at week 29 and at week 31 (**Fig. 2B**). The number of CD4⁺ and CD8⁺ T cells of saline-treated mice was not different to all groups of irinotecan-treated animals both at 29 and 31 weeks of age (**Fig. 2C,D**). Further looking at the activity of T and B cells, we found a significant reduction of T cell activation state (expressed as CD4⁺CD69⁺ cells) only in mice treated with 12.5

mg/kg irinotecan but not in other irinotecan-treated groups at week 29 (**Fig. 3A**). At week 31 a decline in CD4+CD69+ cells was observed only in the groups treated with 6.25 and 25 mg/kg irinotecan (**Fig. 3A**). Remarkably, all irinotecan-treated mice showed a higher number of CD4+CD69+ cells than age-matched Balb/c mice or NZB/W F1 mice at week 8 of age. B cell activation, which was determined by the number of IgG-secreting splenocytes, was lower only in the group treated with the highest dose of irinotecan at week 29 (**Fig. 3B**) consistent with previously published data (14). No differences between saline-treated and all irinotecan-treated groups were seen at week 31 (**Fig. 3B**). Moreover, B cell activation of all irinotecan-treated groups at either week 29 or week 31 was not lower than that of age-matched Balb/c mice. Based on lymphocyte numbers as well as B and T cell activity assays, there was no evidence for a significant immune suppression caused by irinotecan. This was confirmed by measurement of anti-dsDNA antibodies and total IgG in the plasma of lupus-prone mice showing no differences between saline- and irinotecan-treated groups (**Fig. 3C,D**).

DNA sensing is not involved in irinotecan-mediated suppression of lupus nephritis

According to our previous work (14) and the data presented here, immunosuppression does not seem to play an important role in irinotecan-mediated suppression of lupus disease. Hence we looked for alternative mechanisms. As described above, topoisomerase I and its inhibitors are known for modification of genomic DNA with induction of ssDNA or dsDNA breaks depending on the rate of cell division. We, therefore, speculated that treatment with irinotecan produced ssDNA breaks potentially in circulating DNA. Since DNA sensing pathways require dsDNA exclusively (26), the induction of ssDNA might affect DNA sensing-mediated systemic

inflammation. Because type I interferon production is characteristic of DNA sensing-induced systemic inflammation (27), IFN- β levels in splenocytes of NZB/W F1 mice treated with irinotecan were measured. As a result, no differences were found in the IFN- β levels of saline- and irinotecan-treated mice neither at week 29 nor at week 31 (**Suppl. Fig. 1A,B**) suggesting that inhibition of DNA sensing is not involved in irinotecan-mediated suppression of SLE.

Topoisomerase I-induced DNA relaxation enhance binding of anti-dsDNA antibodies

We next postulated that DNA modified by topoisomerase I and its inhibitor might change binding of anti-dsDNA antibodies to DNA (26). We, therefore, incubated different types of dsDNA with recombinant topoisomerase I alone or in combination with the topoisomerase I inhibitor camptothecin and measured binding of anti-dsDNA antibodies. Unexpectedly, topoisomerase I alone increased binding of anti-dsDNA antibodies from plasma of lupus-prone NZB/W F1 mice and from sera of patients with SLE to calf thymus (ct) DNA (**Fig. 4A,B**). Similar effects were shown for nucleosomal DNA (**Suppl. Fig. 2A,B**) and for AT-rich DNA (**Suppl. Fig. 2C,D**). The concentration of topoisomerase required to mediate an augmentation of dsDNA binding was 10 times less than the amount of DNA. When omitting dsDNA in these assays some increased binding was seen only when using the highest concentration of topoisomerase I (**Fig. 4A,B**) excluding a contribution of anti-topoisomerase I antibodies reported to be present in lupus patients (28, 29). Moreover, when applying for detection the monoclonal anti-dsDNA antibody HYB331, a more than 20-fold increase in binding of topoisomerase I-treated ctDNA was observed (**Fig. 4C**) compared to an approx. 3-fold increase when using polyclonal mouse or human sera in similar ELISA settings as shown above.

Since topoisomerase I alone mediates DNA relaxation (30), we further determined whether increased anti-dsDNA binding correlates with the amount of relaxed DNA. Supercoiled pBR322 plasmid DNA was incubated with increasing concentrations of recombinant topoisomerase I and DNA relaxation was visualized by gel electrophoresis (**Fig. 5A**). In addition, modified pBR322 plasmid DNA was used for the measurement of anti-dsDNA binding (**Fig. 5B**) demonstrating a correlation between DNA relaxation and enhanced binding of anti-dsDNA antibodies. When adding camptothecin, an *in vitro* active topoisomerase I inhibitor (irinotecan itself is an inactive prodrug which has to be converted *in vivo* into its active form), the effect of topoisomerase I on dsDNA binding was reversed. Camptothecin in a concentration $\geq 2.5 \mu\text{M}$ significantly decreased the binding of anti-dsDNA antibodies from both mice and humans to ctDNA (**Fig. 5C,D**). Inhibitors of topoisomerase I might therefore suppress murine or potentially human lupus by diminishing the binding of anti-dsDNA antibodies and by decreasing pro-inflammatory immune complexes.

Further reduced dosages of irinotecan ameliorate established lupus nephritis and prolong survival

To further investigate the efficacy of irinotecan in the treatment of lupus, we performed experiments using yet another reduced dose of irinotecan in the treatment of established lupus nephritis. Application of irinotecan to NZB/W F1 mice was initiated at the beginning of the disease defined as proteinuria \geq grade 3+ measured twice, one week apart. Irinotecan 1 mg/kg was given thrice a week. After one treatment cycle both saline- and irinotecan-treated mice received no further treatment for at least 3 weeks. Relapsing disease was diagnosed and treated as initial disease. This treatment schedule demonstrated a reversal of established proteinuria in irinotecan-treated mice at the second and third week after initiation of the treatment

(**Fig. 6A**). While the body weight of saline-treated control mice declined from week 29, the body weight of irinotecan-treated mice was stable until week 38 of age. A significant difference in body weight was observed from week 34 until week 39 of age (**Fig. 6B**). Moreover, treatment significantly prolonged survival of irinotecan-treated mice ($P=0.0064$, **Fig. 6C**).

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Discussion

Our data presented here show that lupus in NZB/W F1 mice can be treated with remarkably reduced doses of the topoisomerase I inhibitor irinotecan. The minimal dose applied in our experiments for the treatment of established lupus nephritis was more than 50 times lower than the dose normally used for chemotherapy in humans. The calculation was as follows: In Europe, for chemotherapy in humans a dose of 350 mg/m² is given every third week (21, 31). In lupus-prone mice a dose of 3 mg/kg per week was applied for the treatment of established disease. According to the grade of proteinuria this treatment was allowed to be repeated every fourth week. The dose of 3 mg/kg in mice corresponds to 9 mg/m² in humans, for conversion see <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm078932.pdf>. Calculated for 12 weeks, a total irinotecan dose of 1400 mg/m² are applied in humans, while the experimental plan for NZB/W F1 allowed a total dose of 27 mg/m² during the same period of time. It is also worth mentioning that the dose of irinotecan used in anti-cancer experiments in mice varies between 100 and 200 mg/kg per week (32-34). These numbers indicate that the molecular pathways targeted by irinotecan for chemotherapy and for suppression of SLE are not similar. Whereas our previous data suggested a mild and transient immunosuppression using high dose irinotecan (14), our current results with low dose irinotecan showed an amelioration of lupus-associated B cell hyper-responsiveness, but provide no evidence of immunosuppression. Instead we found evidence that irinotecan-mediated effects on lupus may occur through modification of DNA. The idea to target DNA is not new because circulating DNA is believed to be a major pathogen in SLE (35). One approach has been the use of recombinant DNase I in order to destroy circulating DNA. The results of these experiments were conflicting

(36, 37), perhaps reflecting the recently reported protection of circulating DNA from extracellular degradation by certain peptides produced as neutrophil extracellular traps in patients with SLE (38). If extracellular DNA is difficult to eliminate, an alternative possibility is to manipulate the DNA in order to make it less antigenic. As a consequence, modified circulating DNA can transform the binding of extracellular DNA to renal structures as it was shown before for the application of the glucosaminoglycan heparin (39). Alternatively, modified DNA might also reduce the binding of anti-dsDNA antibodies to the DNA and so lead to fewer immune complexes. We hypothesize, based on the data presented here, that irinotecan reverses the increased binding of DNA to anti-dsDNA antibodies that is mediated by topoisomerase I. The question is how topoisomerase I facilitates enhanced binding of anti-dsDNA antibodies to the DNA? Preferential binding of anti-dsDNA antibodies to a protein-DNA complex is unlikely because binding of topoisomerase I to the DNA is transient (40). Alternatively, the induction of ssDNA breaks as the underlying mechanism for enhanced anti-dsDNA binding is also not very likely since without its inhibitor topoisomerase I cleaves and subsequently re-joins dsDNA leaving intact strands. A third possibility is a change in DNA confirmation which is supported by our data showing that anti-dsDNA antibodies increase their binding to dsDNA after topoisomerase I has catalyzed relaxation of the DNA. This suggests that anti-dsDNA antibodies prefer binding to relaxed DNA. Accordingly, enhanced binding of anti-dsDNA antibodies to topoisomerase I-modified dsDNA was prevented when applying the topoisomerase I inhibitor camptothecin. It is known that topoisomerase I inhibitors inhibit topoisomerase I-mediated DNA relaxation (41, 42). Consequently, we suppose that inhibition of DNA relaxation could explain how irinotecan influences the course of SLE. Interestingly, inhibition of DNA relaxation by topoisomerase I inhibitors was reported to preferentially affect negatively supercoiled DNA and to omit positive

supercoils (43). Whether this has an impact on irinotecan-mediated suppression of SLE remains unknown at the moment.

The next question is where topoisomerase I induces relaxation of circulating DNA. Assuming that this effect indeed occurs *in vivo*, it may be that DNA is modified intracellularly and then released by apoptotic cells as it is believed to be the mechanism for the generation of circulating DNA in SLE (44). Alternatively, modification of DNA might arise in the blood presuming the presence of extracellular topoisomerase I. While topoisomerase I contains several nuclear localization signals at the N-terminus and seems to be localized exclusively in intracellular DNA-containing structures, e.g. the nucleus and mitochondria (45), there is some evidence that topoisomerase I can get access to the extracellular space under certain circumstances. Otherwise it would be not explainable how anti-topoisomerase I antibodies arise in SLE or other autoimmune diseases (28, 46).

Based on the data provided here we are planning a first clinical trial where patients suffering from active lupus nephritis refractory to conventional medication will be treated with low dose irinotecan. If low dose irinotecan will show the same efficacy during the trial as we have shown here in lupus-prone mice, undesired side effects known from conventional immunosuppressive therapy (47) might disappear. However, in the context of side effects concerns may raise whether irinotecan can be given to patients with impaired renal function. The answer is favorable since detoxification and elimination of irinotecan respectively of its active metabolite SN-38 is facilitated by glucuronidation by uridine diphosphate-glucuronosyltransferase 1A in the liver (48). Therefore, irinotecan can be used in patients with renal failure without the risk of increased side effects (49).

In conclusion, given our data in lupus-prone mice treated with low dose irinotecan and providing that topoisomerase I is a highly conserved enzyme showing 96%

homology between mouse and men (50), low dose irinotecan may be a valuable therapeutic option for patients with lupus nephritis and potentially with other SLE manifestations.

For Peer Review

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Figure legends

Figure 1

Reduced dosages of irinotecan suppress lupus nephritis and prolong survival in NZB/W F1 mice. NZB/W F1 mice were treated thrice a week from week 24 of age with the indicated dosages of irinotecan. Treatment was repeated every fourth week. $n=10$ for all groups. **(A)** Proteinuria was measured once a week using Albustix®. The onset of the disease was defined as proteinuria grade 4+ (≥ 2000 mg/dL) measured twice, one week apart. Saline-treated mice showed a significant higher frequency of grade 4+ proteinuria compared to all irinotecan-treated groups from week 40 until week 52 of age. After week 53 of age only dosages of ≥ 6.25 mg/kg irinotecan significantly diminished grade 4+ proteinuria. Two-way ANOVA. **(B)** Body weight of all irinotecan-treated groups remained stable until week 58 of age and showed a significant difference from week 42 until week 64 of age compared to the saline-treated control group. Two-way ANOVA. **(C)** Animals were sacrificed due to the severity of the disease. Improved survival of all irinotecan-treated groups compared to saline-treated mice. Mantel-Cox log-rank test. $P<0.0001$.

Figure 2

Treatment with low dose irinotecan is not accompanied by a reduction of T and B lymphocytes. NZB/W F1 mice were treated from week 24 with the indicated dosages of irinotecan or saline. Application of irinotecan was performed thrice a week. Treatment was repeated every fourth week. Mice were sacrificed 1 and 3 weeks after the second treatment cycle (indicated as week 29 and week 31 of age). $n=14$. As control groups Balb/c mice 30 weeks of age ($n=10$) and NZB/W F1 mice 8 weeks of age ($n=20$) and 23 weeks of age ($n=14$) were used. **(A)** Spleen weight was

determined directly after sacrifice. One-way ANOVA. $*P<0.05$, $**P<0.01$. Flow cytometry analysis demonstrated the number of B cells (**B**), the number of CD4+ cells (**C**) and the number of CD8+ cells (**D**). One-way ANOVA.

Figure 3

Low dose irinotecan corrects lupus-associated overstimulation of the immune system but does not induce immunosuppression. NZB/W F1 mice were treated from week 24 of age with the indicated dosages of irinotecan or saline. Application of irinotecan was performed thrice a week. Treatment was repeated every fourth week. Mice were sacrificed 1 and 3 weeks after the second treatment cycle (indicated as week 29 and week 31 of age). $n=14$. As control groups Balb/c mice 30 weeks of age ($n=10$) and NZB/W F1 mice 8 weeks of age ($n=20$) and 23 weeks of age ($n=14$) were used. T cell activation was determined by flow cytometry measuring CD4+CD69+ cells (**A**). The activity of B cells was determined by ELISpot assessing the number of IgG secreting cells (**B**). Anti-dsDNA antibodies (**C**) and total IgG (**D**) in the blood plasma of mice were measured by ELISA assay. One-way ANOVA. $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$.

Figure 4

Topoisomerase I induces increased binding of anti-dsDNA antibodies to ds ctDNA. Fifty μg per ml of DNA were treated with the indicated concentrations of recombinant topoisomerase I for 30 min at 37°C and then incubated on 'maxisorp' plates at 4° overnight. Bound anti-dsDNA antibodies from (**A**) plasma of lupus-prone mice ($n=10$) or from (**B**) sera of lupus patients ($n=14$) or using (**C**) the monoclonal antibody HYB331 were determined by ELISA. Values are expressed as fold increase of the respective control treated with the same amount of BSA. Two-way ANOVA.

**** $P < 0.0001$. Representative results of two or three independent experiments for polyclonal sera. Mean \pm SD of three independent experiments with duplicates for HYB331.

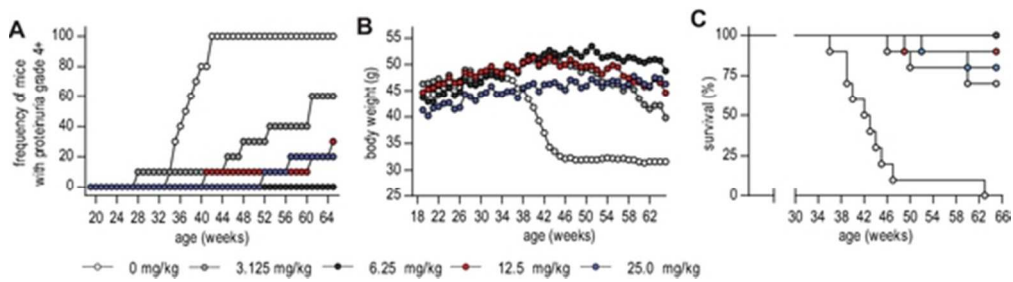
Figure 5

DNA relaxation correlates with enhanced binding of anti-dsDNA antibodies and inhibition of this effect by addition of the topoisomerase I inhibitor camptothecin. **(A)** Fifty $\mu\text{g/ml}$ of DNA supercoiled pBR322 plasmid DNA was incubated with the indicated concentrations of topoisomerase I for 1 h at 37°C. DNA relaxation was visualized on a 1% agarose gel. **(B)** Binding of murine anti-dsDNA antibodies to supercoiled DNA treated with topoisomerase I was determined by ELISA. Values are expressed as fold increase of the respective control treated with the same amount of BSA. **(C)** and **(D)** Addition of the topoisomerase I inhibitor camptothecin inhibited enhanced binding of anti-dsDNA antibodies to topoisomerase I-modified ctDNA. Fifty $\mu\text{g/ml}$ of DNA was incubated with 75 ng per μg topoisomerase I, the indicated concentrations of camptothecin and 10% DMSO for 1 h at 37°C. After incubation with murine plasma or human sera bound antibodies were measured. Values are expressed as fold increase of the respective control treated with the same concentrations of BSA and camptothecin. One-way ANOVA. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$. Representative results of two independent experiments.

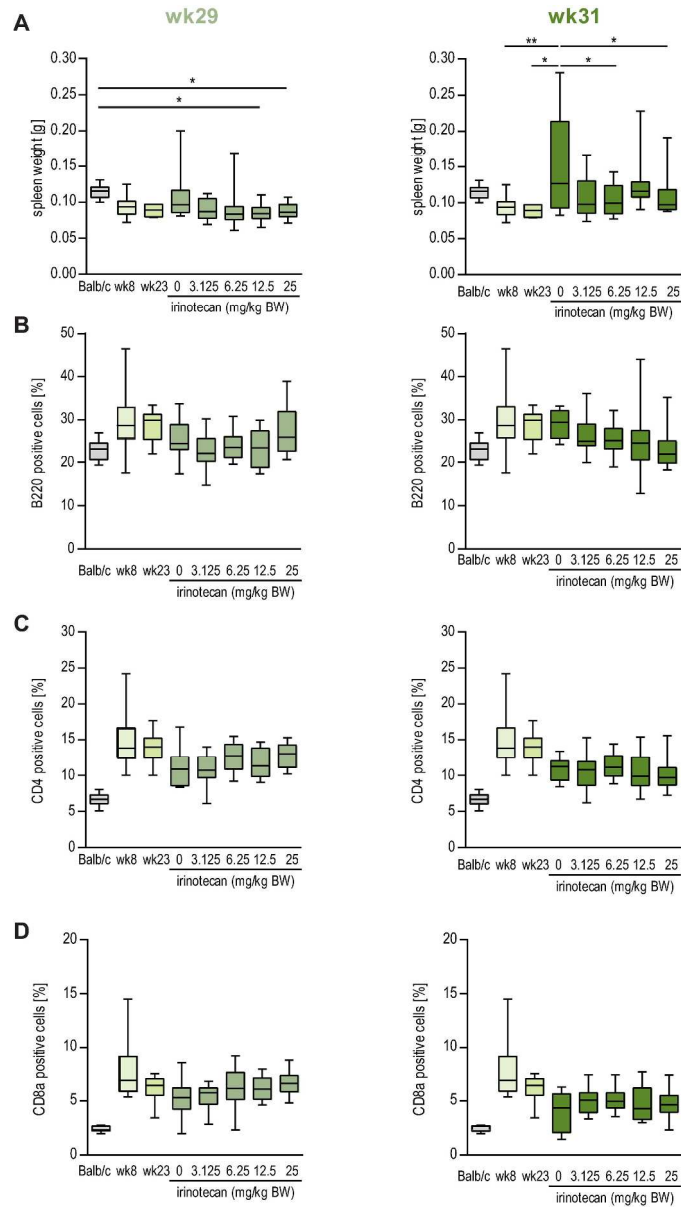
Figure 6

An irinotecan dose which is more than 50 times lower than the dose used for chemotherapy reverses established lupus nephritis in NZB/W F1 mice. Mice were treated from established proteinuria which was defined as \geq grade 3+ (300 mg/dL) measured twice one week apart. Irinotecan at a dose of 1 mg/kg was applied thrice a

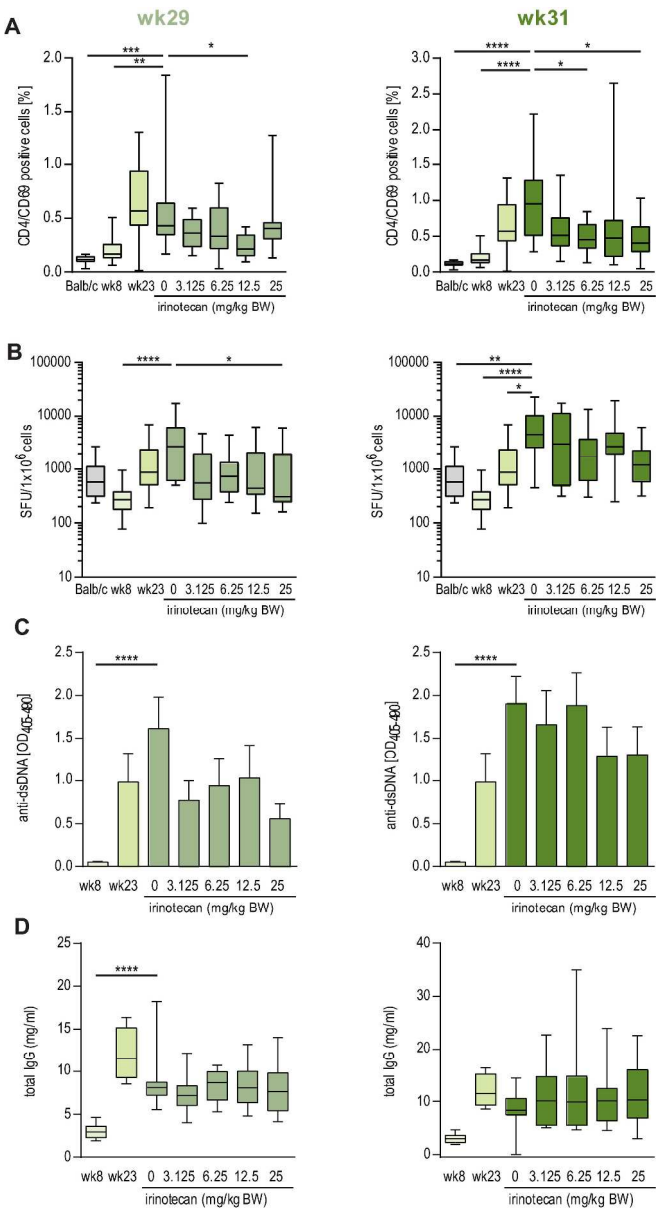
week. Further treatments were given according to the grade of proteinuria as described for the initial treatment initiation; between two cycles a treatment stop of at least 3 weeks was defined. **(A)** Reversal of established proteinuria determined after the first treatment cycle. $n=9$ for saline- and $n=10$ for irinotecan-treated mice. Two-way ANOVA. $*P<0.05$, $**P<0.01$. **(B)** Body weight of irinotecan- and saline-treated mice. $n=10$ for both groups. Two-way ANOVA. $P<0.05$ from week 34 until week 39 of age. **(C)** Survival of both saline- and irinotecan-treated groups; $n=10$ for both groups. Animals were sacrificed due to the severity of the disease which was defined as a proteinuria grade 4+ (2000 mg/dL) and a weight loss of >25% calculated from the beginning of the disease. Mantel-Cox log-rank test. $P=0.0064$.



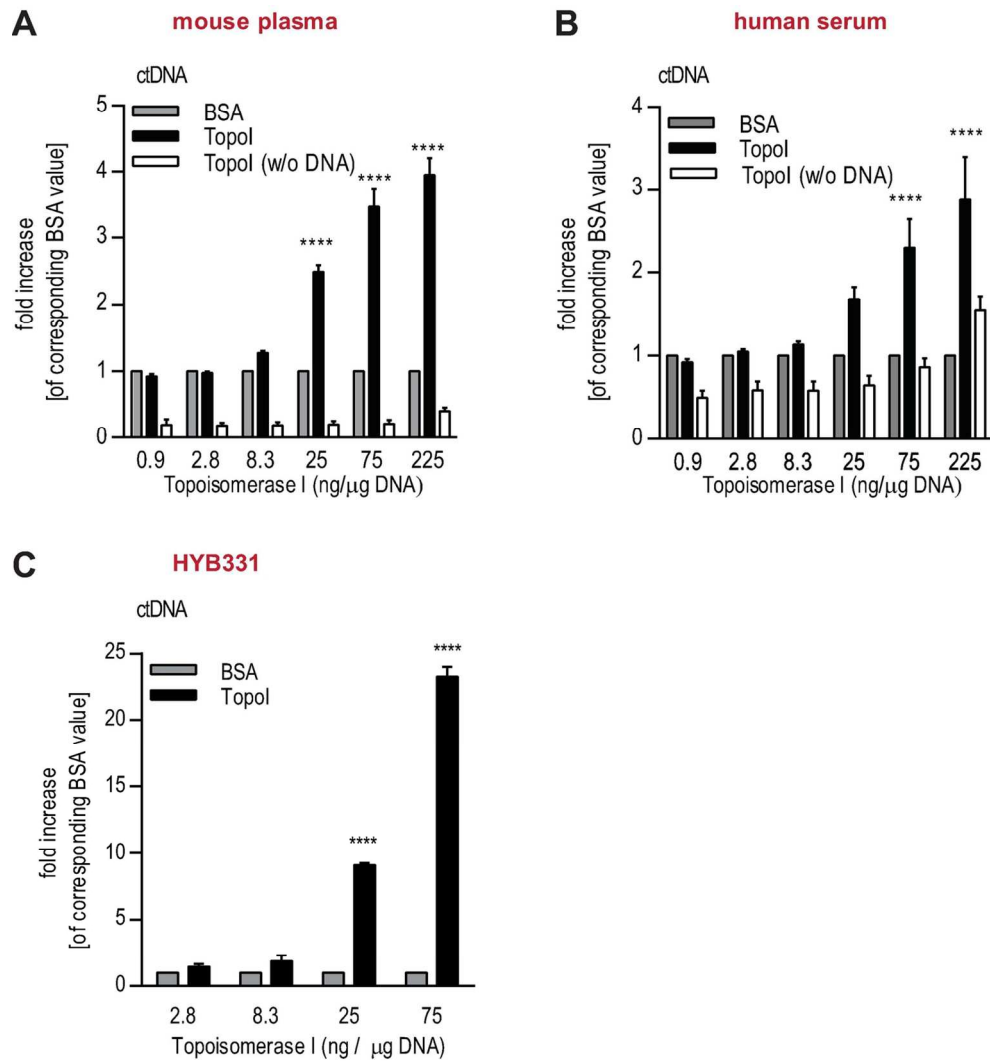
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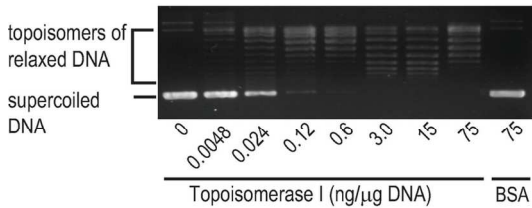


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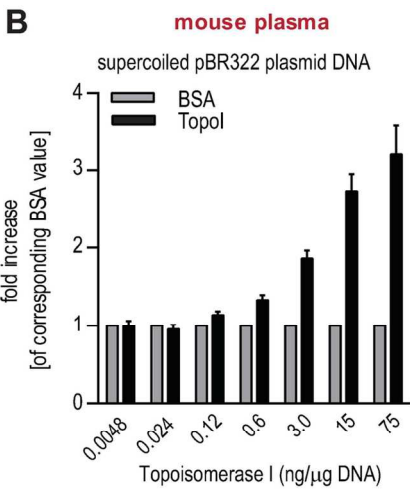


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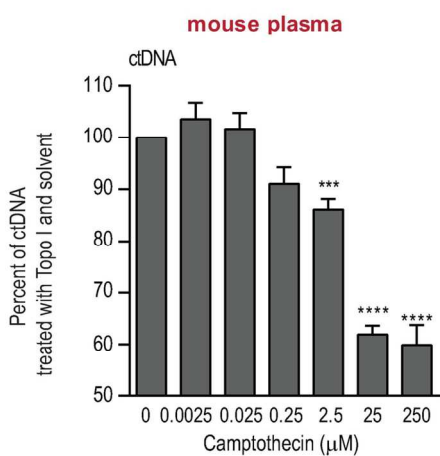
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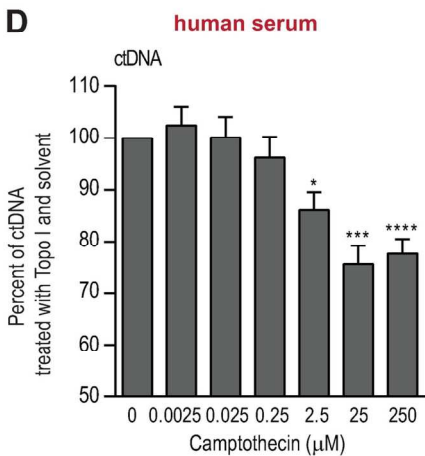
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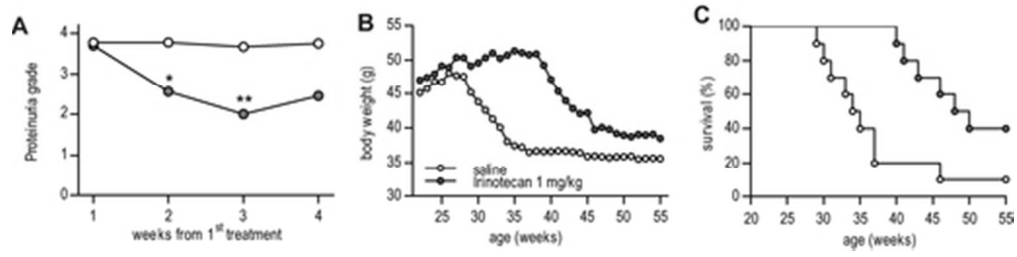
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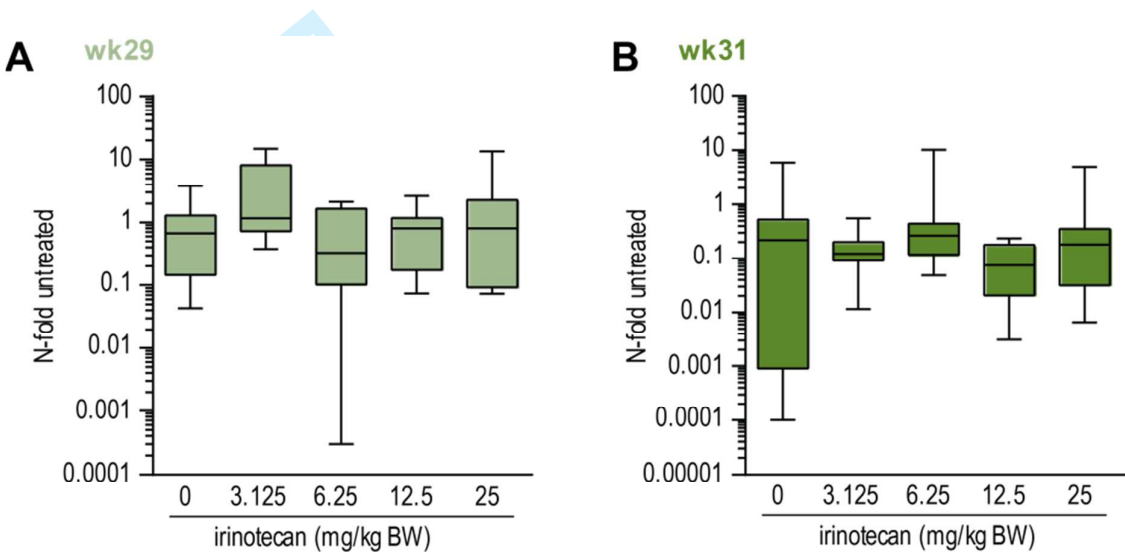
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SUPPLEMENT

Frese-Schaper et.al.

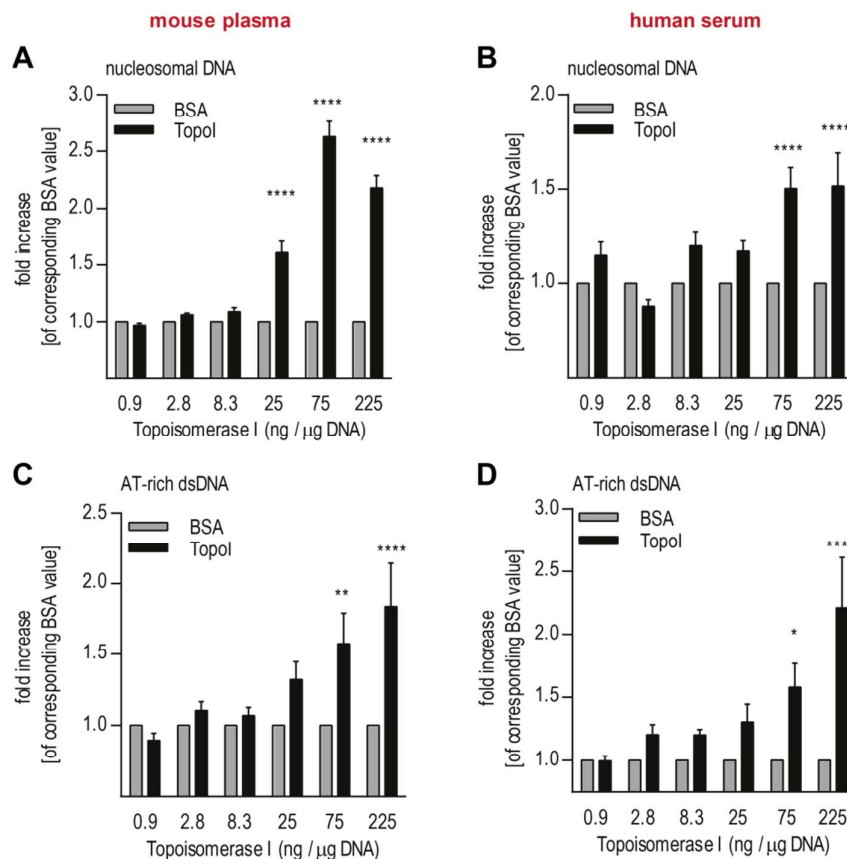
Low dose irinotecan improves advanced lupus nephritis in mice potentially by changing DNA relaxation and anti-dsDNA binding

Supplementary Figure 1



DNA sensing is not involved in irinotecan-mediated suppression of lupus nephritis. Type I interferon IFN- β mRNA levels were determined in splenocytes of lupus-prone NZB/W F1 mice at 29 and 31 weeks of age which were treated with irinotecan using the indicated concentrations. n=8 per group. INF- β mRNA levels were determined by quantitative PCR. Data were normalized against the levels of β -actin which was used as internal control. Statistics was performed with Kruskal–Wallis test.

Supplementary Figure 2



Topoisomerase I induces increased binding of anti-dsDNA antibodies to ds nucleosomal or AT-rich DNA. Fifty μ g per ml of DNA were treated with the indicated concentrations of recombinant topoisomerase I for 30 min at 37°C and then incubated on 'maxisorp' plates at 4° overnight. Bound anti-dsDNA antibodies from plasma of lupus-prone mice (n=10) or sera of lupus patients (n=14) were determined by ELISA. Values are expressed as fold increase of the respective control treated with the same amount of BSA. Two-way ANOVA. *P<0.05, **P<0.01, ****P<0.0001. Representative results of two or three independent experiments.